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Tamoxifen-Inducible Form of Cre: A Tool for Temporally Regulated Gene Activation/Inactivation in the Mouse

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In recent years, the Cre integrase from bacteriophage P1 has become an essential tool for conditional gene activation and/or inactivation in mouse. In an earlier report, we described a fusion protein between Cre and a mutated form of the ligand binding domain of the estrogen receptor (Cre-ER^{TM}) that renders Cre activity tamoxifen (TM) inducible, allowing for conditional modification of gene activity in the mammalian neural tube *in utero*. In the current work, we have generated a transgenic mouse line in which *Cre-ER^{TM}* is ubiquitously expressed to permit temporally regulated Cre-mediated recombination in diverse tissues of the mouse at embryonic and adult stages. We demonstrate that a single, intraperitoneal injection of TM into a pregnant mouse at 8.5 days postcoitum leads to detectable recombination in the developing embryo within 6 h of injection and efficient recombination of a reporter gene in derivatives of all three germ layers within 24 h of injection. In addition, by varying the dose of TM injected, the percentage of cells undergoing a recombination event in the embryo can be controlled. Dose-dependent excision induced by TM was also possible in diverse tissues in the adult mouse, including the central nervous system, and in cultured cells derived from the transgenic mouse line. This inducible Cre system will be a broadly useful tool to modulate gene activity in mouse embryos, adults, and culture systems where temporal control is an important consideration.

Key Words: **Cre; Cre-ER^{TM}; gene activation; inactivation; inducible; estrogen receptor; tamoxifen; 4-hydroxy-tamoxifen; mouse; bacteriophage P1.**

INTRODUCTION

The use of the P1 phage-derived integrase Cre to catalyze recombination between its loxP target sites has revolutionized the analysis of gene function in the mouse (Nagy, 2000; Rossant and McMahon, 1999; Rossant and Nagy, 1995). One powerful use of this technology is in the conditional removal or activation of gene function. In the former, Cre-mediated recombination leads to the precise excision of an essential region within a gene so that a functional product is not produced. In the latter, Cre-mediated recombination removes a functional barrier to the production of an active gene product, thereby switching on gene activity. One way in which the utility of this approach can be enhanced is by developing ways in which Cre activity can be controlled.

A number of groups have described various approaches to controlling the spatial and/or temporal expression of the enzyme (Gorman and Bullock, 2000; Nagy, 2000; Rossant and McMahon, 1999). In one of these, a fusion gene is created between Cre and a mutant form of the ligand-binding domain of the estrogen receptor (ER^{TM}). This mutation prevents binding of its natural ligand (17β-estradiol) at normal physiological concentrations, but renders the ER^{TM} domain responsive to 4-hydroxy (OH)-TM (Danielian *et al.*, 1993; Fawell *et al.*, 1990; Littlewood *et al.*, 1995). Fusion of Cre with ER^{TM} leads to the ER^{TM}-dependent cytoplasmic sequestration of Cre by Hsp90 (Mattioni *et al.*, 1994; Picard, 1994), thereby preventing Cre-mediated recombination, a nuclear event. However, binding of 4OH-TM leads to a disruption of the interaction with

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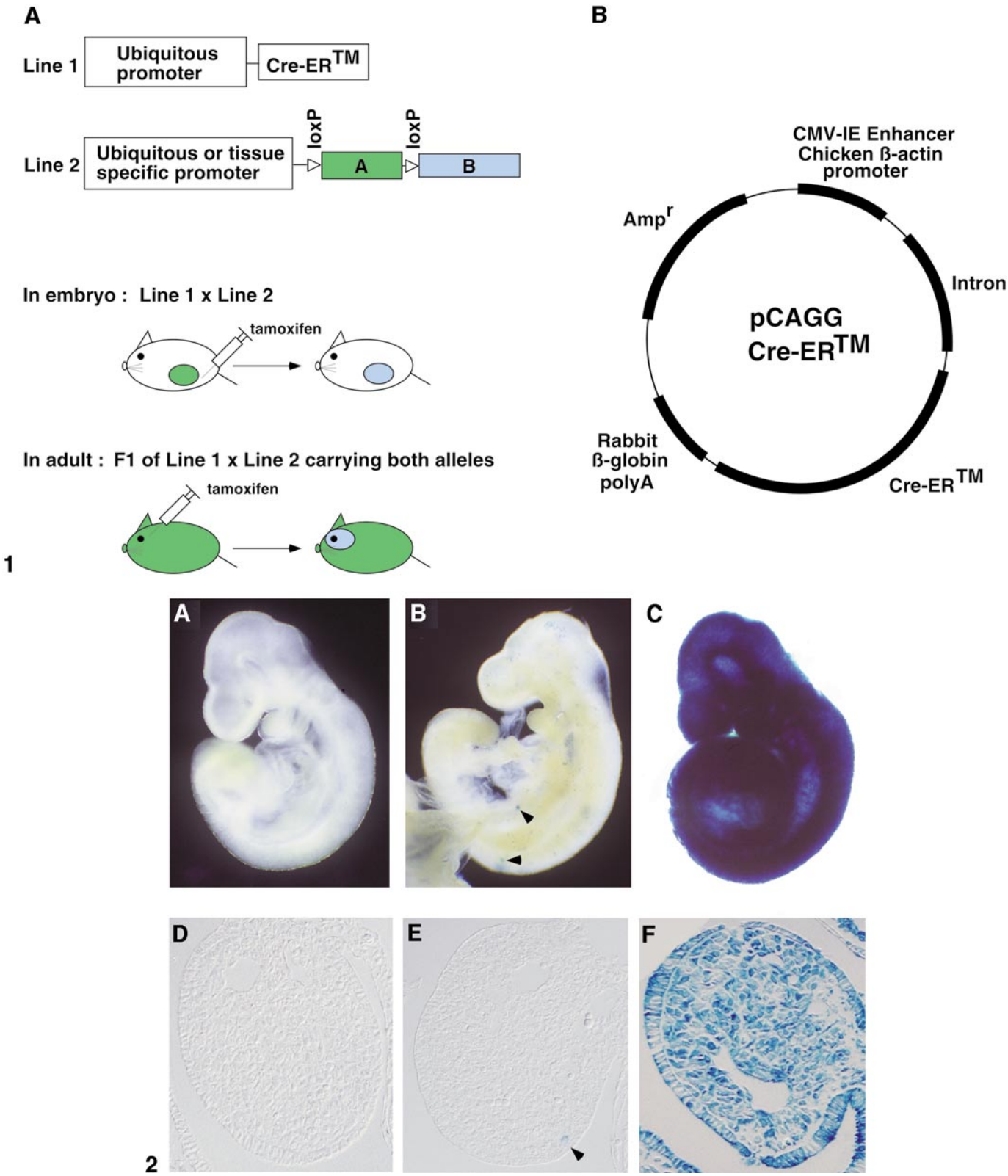


FIG. 1. Development of a TM-inducible gene modification system. (A) Potential use of *CAGGCre-ER^{TM}* line to regulate gene activation or inactivation in an embryo or postnatal animal. *CAGGCre-ER^{TM}* line (line 1) can be used to excise DNA sequences flanked by two loxP

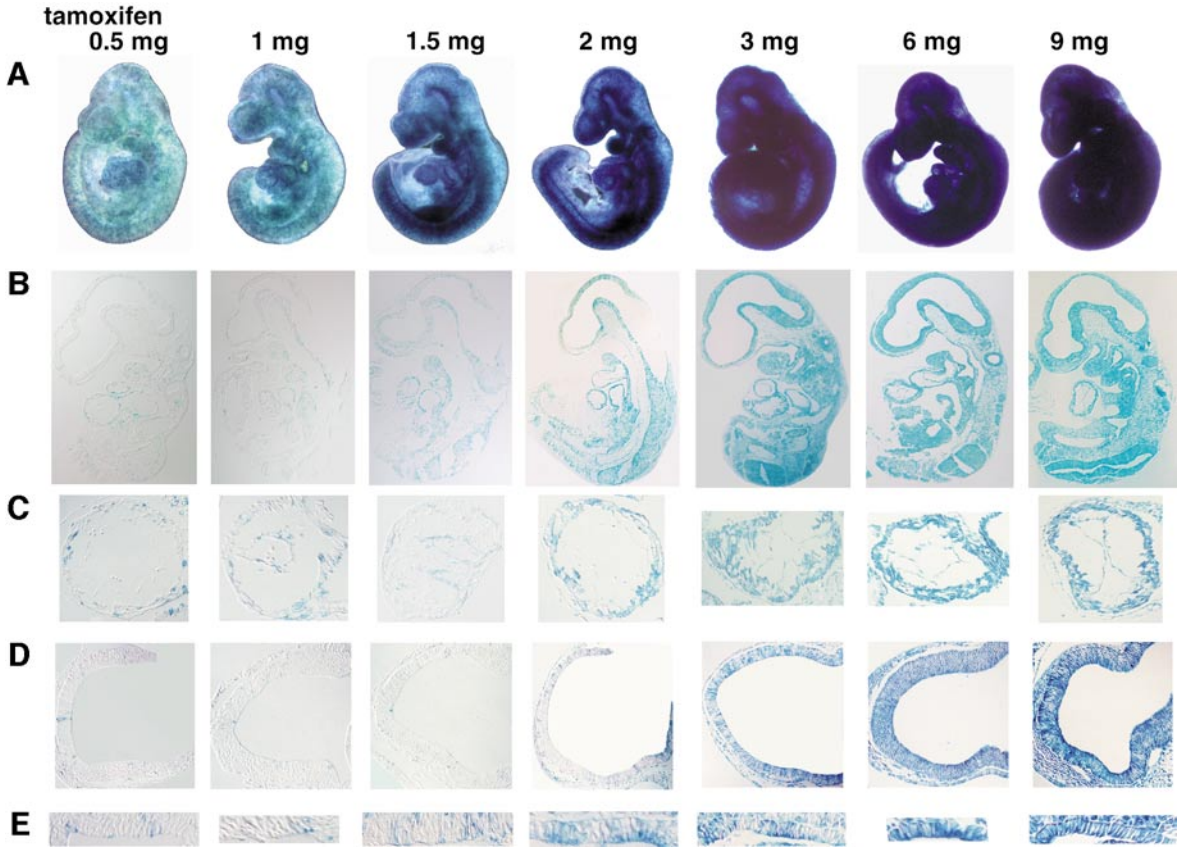


FIG. 3. TM induces dose-dependent recombination in *CAGGCre-ER^{TM}; R26R* mouse embryos. Compound transgenic embryos (*CAGGCre-ER^{TM}; R26R*) were subjected to whole-mount histochemical staining for β-galactosidase activity at 9.5 dpc, 24 h after administration of the indicated dose of TM administration. (A) Whole-mount views of embryos. (B–E) Nomarski images of 6-μm sections through embryos in (A). (B) Sagittal view of embryo, (C) heart, (D) telencephalon, and (E) gut. Dose-dependent recombination was observed in tissue derivatives of all three germ layers.

Hsp90, permitting access of Cre-ER^{TM} to the nucleus and initiation of recombination.

We (Danielian *et al.*, 1998) and others (Brocard *et al.*, 1997; Feil *et al.*, 1996; Imai *et al.*, 2001; Indra *et al.*, 1999; Schwenk *et al.*, 1998; Vasioukhin *et al.*, 1999; Vooijs *et al.*, 2001) have demonstrated tissue-specific manipulation of gene activity by using transgenic *Cre-ER^{TM}* lines. Importantly, these approaches have not been restricted to the adult mouse: injection of TM into the pregnant animal has been shown to activate recombination in the developing

sites (line 2). Excision in an embryo can be accomplished by injection of TM into a pregnant mother that carries double transgenic embryos for lines 1 and 2. In postnatal animals, excision is achieved either ubiquitously by intraperitoneal injection of TM or within a specific target organ by local injection of 4OH-TM. In this figure, an example of the local excision is shown. (B) Map of the pCAGGCre-ER^{TM} construct. Expression of Cre-ER^{TM} is driven by a chimeric promoter/enhancer of the cytomegalovirus immediate-early enhancer and the chicken β-globin promoter/enhancer. The pCAGGs vector also contains an intron from chicken β-globin and a polyadenylation signal from the rabbit β-globin.

FIG. 2. Efficient recombination in *CAGGCre-ER^{TM}; R26R* mouse embryos following administration of TM at 8.5 dpc. Whole-mount views of 9.5-dpc embryos following histochemical staining of embryos for β-galactosidase activity. (A) Nontransgenic embryo. (B) *CAGGCre-ER^{TM}; R26R* compound transgenic embryo 24 h after corn oil injection. Note that there are some lacZ-positive cells in the absence of TM induction (arrowhead in B). (C) *CAGGCre-ER^{TM}; R26R* compound transgenic embryo 24 h after injection of 3 mg of TM (in corn oil)/40 g of mother's body weight induces widespread recombination. (D–F) Nomarski image of a 6-μm section through the branchial arch region of embryos in (A–C), respectively. In (E), cells displaying β-galactosidase activity are indicated by the arrowhead.

embryo (Danielian *et al.*, 1998; Kimmel *et al.*, 2000; Vallier *et al.*, 2001). However, the tissue specificity of these lines, while well suited to certain types of studies, limits the general utility of these transgenic strains.

To create a more broadly useful strain of mouse, we set out to generate a line in which *Cre-ER*^{TM} is ubiquitously expressed (Fig. 1A). In principle, crossing these mice to an appropriate target strain (Fig. 1A) would permit TM-dependent recombination in all tissues, with precise temporal control, at embryonic and adult stages. We report a line that exhibits widespread expression of *Cre-ER*^{TM} and demonstrate the utility of this strain in the control of Cre-mediated genetic modification in embryonic and adult mice and in cell culture.

EXPERIMENTAL METHODS

Generation of Construct

A fragment encoding Cre-ER^{TM} (Danielian *et al.*, 1998) was cloned into pCAGGS vector (Niwa *et al.*, 1991) to generate pCAGGCre-ER^{TM}. The *Apa*I site of pBS-Cre-ER^{TM} was replaced by an *Eco*RI site by using the oligo (CGAATTCGGGCC). An *Eco*RI fragment containing Cre-ER^{TM} was then subcloned into the *Eco*RI site of pCAGGS. The orientation of the gene was confirmed by sequencing.

Generation of Transgenic Animals

For pronuclear injection, pCAGGCre-ER^{TM} was digested with *Sal*I and *Hin*dIII to remove vector sequences. The insert was isolated by agarose gel electrophoresis and purified by electroelution, and then by using a PCR purification kit (Qiagen). The purified DNA was resuspended in 10 mM Tris, pH 7.5, 0.1 mM EDTA. The DNA concentration was determined by UV spectrophotometry and the quality confirmed by agarose gel electrophoresis. Purified DNA was subjected to pronuclear injection by using a standard method (Hogan *et al.*, 1994). Potential founder animals were screened by polymerase chain reaction (PCR) and further confirmed by Southern blotting analysis. For PCR, mouse ear DNA was amplified by 35 cycles on a thermal cycler using primers from CAGGs (CTCTAGAGCCTCTGCTAACC) and Cre (CCTGGCGATCCCTGAACATGTCC) sequence. The size of the amplified product is approximately 400 bp.

Cross-Breeding of Mice

Male CAGGCre-ER^{TM} transgenics were crossed to females that carried the Rosa reporter allele, R26R (Soriano, 1999). Offspring were genotyped by PCR for CAGGCre-ER^{TM} (as above) and R26R alleles (Soriano, 1999), and males that carried both genetic modifications were crossed to Swiss-Webster (SW; Taconic) females to obtain double transgenic embryos (at a predicted frequency of 1 in 4) for *in utero* studies.

Induction with TM or 4OH-TM

TM (Sigma) was dissolved in corn oil (Sigma) at a concentration of 10 mg/ml. For the initial screening of the transgenic founders, 2 mg of TM was injected intraperitoneally into pregnant mothers at embryonic day 10.5 (E10.5). Forty-eight hours later, embryos were fixed and subjected to 5-bromo-4-chloro-3-indolyl-D-galactoside (X-gal) staining as described below. For detailed characterization of the Cre activity, 0.5, 1, 1.5, 2, 3, 6, or 9 mg of TM/40 g of body weight was injected into pregnant mothers at 8.5 days postcoitum (dpc) and embryos were harvested for 3, 6, 12, or 24 h postinjection and subjected to histochemical staining to visualize β-galactosidase activity. For analysis of adults, corn oil alone or 3 or 9 mg of TM/40 g of body weight was injected intraperitoneally for 5 consecutive days. Five days after the last injection, various organs from the mice were fixed and subjected to X-gal staining. Single dose study was carried out by injecting 9 mg of TM, and various organs were subjected to histochemical staining 24 h or 5 days after the injection. Unless specified otherwise, all injections of TM are per 40 g body weight. 4OH-TM (Sigma) was dissolved in ethanol to make a 1 mM stock solution and further diluted to appropriate concentrations prior to use.

X-Gal Staining, Histology, and Immunohistochemistry

Embryos collected at 9.5 dpc were stained postfixation in X-gal for 3 h at 37°C (Whiting *et al.*, 1991). Following X-gal staining, embryos were refixed, dehydrated, embedded in paraffin, and sectioned at 6 μm. Adult mice were initially perfused with 2% paraformaldehyde, and their organs were dissected and further fixed overnight at 4°C. After washing with PBS several times, organs were cryoprotected with 30% sucrose and embedded in OCT compound (Tissue-Tek). Frozen sections were prepared at 12 μm and subjected to X-gal staining. Immunostaining for Cre-ER protein was carried out by using anti-Cre antibody (Ab) (Babco). Frozen sections from embryos were prepared as described above, then washed with PBS three times, blocked in PBS with 10% sheep serum, 0.1% Triton X-100 for 1 h, and incubated overnight with anti-Cre Ab (1:3000) at 4°C. Sections were washed three times with PBS and the signal was detected by using anti-rabbit-Alexa 488 (Molecular Probes). Counterstaining was carried out by using DAPI to visualize the nucleus. Images were obtained on a Zeiss confocal microscope.

Induction of Recombination by 4OH-TM in Primary Embryo Fibroblast (PEF) Cells and Measurement of β-Galactosidase Activity

PEF cells were obtained from 14.5-dpc embryos as described (Hogan *et al.*, 1994). Embryos were dissected individually and a portion of the tail was used for PCR genotyping to identify *CAGGCre-ER; R26R* embryos as described above. Cultured PEF cells with the correct genotype were passaged once and used for further experiments. Recombination was initiated by adding different doses of 4OH-TM (Sigma) diluted in PEF media. At 6, 12, 24, or 48 h postinduction, PEF cells were subjected to either X-gal staining as described above or an assay for β-galactosidase activity using a high sensitivity β-galactosidase assay kit (Stratagene) following the manufacturer's protocol. The protein concentration of the cell lysate was determined by a BCA protein assay system (Pierce).

RESULTS

Generation of CAGGCre-ER^{TM} Transgenic Mice

To carry out temporally regulated activation or inactivation of gene activity, we sought to generate a transgenic mouse line expressing *Cre-ER^{TM}* (Fig. 1B) in a broad spectrum of cell types at embryonic and adult stages. The promoter driving *Cre-ER^{TM}* expression is a chimeric promoter of the cytomegalovirus immediate-early enhancer and chicken β-actin promoter/enhancer (CAGG) that has been shown to drive widespread expression of a number of genes in transgenic mice (Lobe *et al.*, 1999; Niwa *et al.*, 1991; Pratt *et al.*, 2000). Pronuclear injection of the *CAGGCre-ER^{TM}* generated 33 founder transgenic animals (generation $[G]_0$). Of these, the 17 males were subjected to an assay to verify the transmission of Cre recombination activity into offspring by crossing to R26R reporter females (Soriano, 1999). Pregnant mothers were injected with 2 mg of TM at 10.5 dpc, embryos were collected 48 h later, and β-galactosidase activity was detected with X-gal. Four founders failed to produce any X-gal-positive embryos. Ten lines showed Cre recombinase activity restricted to the heart, somites, and occasionally a portion of the central nervous system (CNS) (data not shown). Two independent lines (lines 3 and 5) generated embryos with β-galactosidase activity in most, if not all, cells (data not shown): one line (line 37) showed patchy β-galactosidase activity throughout the embryo (data not shown). This frequency of obtaining transgenic lines with ubiquitous expression utilizing the CAGG regulatory sequences (2/17) is similar to a previous report (Lobe *et al.*, 1999). Analysis of lines 3 and 5 indicated that the transgenic founders had multiple, independently segregating integration sites. One of the sublines (line 5–8) derived from G_1 progeny of line 5, did not show any further segregation of the transgene insertion and maintained the founder's TM-inducible recombination activity (data not shown). Line 5–8 was chosen for further study.

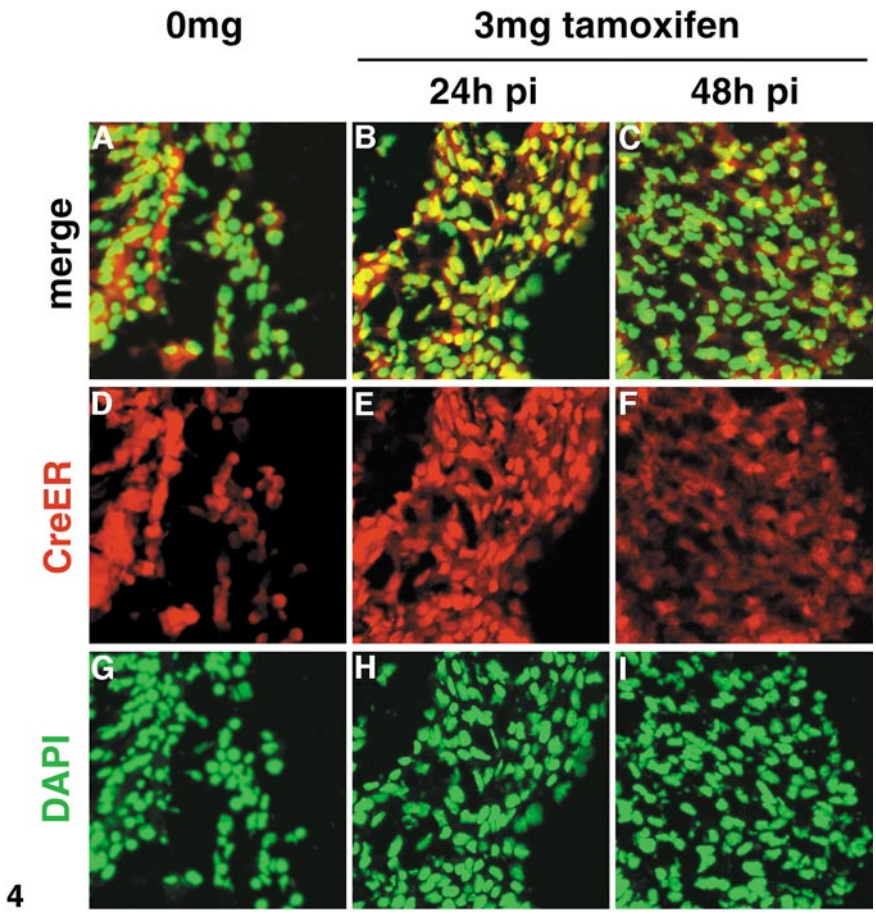
Efficient Cre-Mediated Recombination Induced by TM in Diverse Tissues of the Mouse Embryo

To test the efficiency of TM-dependent Cre recombinase activity in mouse embryos, we focused our analysis at 8.5 dpc. At this time, the three germ layers of the mouse embryo have undergone initial patterning with respect to the major body axes, but most organ systems are not established. Initially, we performed a dose toxicity analysis following a single intraperitoneal injection of TM into nontransgenic SW or B6CBAF1 females at 8.5 dpc. An injection of 3 mg/40 g body weight allowed normal development to term of most embryos (30/48). In contrast, embryos developing in pregnant females injected with higher concentrations (6 and 9 mg/40 g body weight) failed to develop to term, although most embryos were viable at 13.5 dpc (26/36 and 28/44, respectively). The dose toxicities were similar in both strains (data not shown). When these same doses were administered at 11.5 and 14.5 dpc, a good survival frequency to term was observed (6 mg, 33/42 and 36/40, respectively; 9 mg, 21/48 and 30/39, respectively). Importantly, dose toxicity does not appear to show a simple linear correlation with body weight; embryos developing in high mass females (40 g or greater) are able to tolerate higher doses of TM better than those in lower mass females.

In initial studies, *CAGGCre-ER^{TM}; R26R* males were mated to SW females and pregnant females were injected at 8.5 dpc with either corn oil or corn oil supplemented with 3 mg TM/40 g of body weight. Embryos were collected 24 h later and analyzed in whole mount for β-galactosidase activity. No β-galactosidase activity was detected in embryos lacking the R26R transgene (Figs. 2A and 2D) or following corn oil injection (data not shown). Analysis of *CAGGCre- ER^{TM}; R26R* embryos detected a low percentage of cells (estimated at less than 0.1%) that underwent spontaneous, sporadic recombination in the absence of TM (Figs. 2B and 2E). However, 3 mg of TM induced widespread recombination throughout the embryo within 24 h (Fig. 2C). Analysis of tissue sections indicated that greater than 50% of cells expressed the transgenic reporter, indicating

FIG. 4. Intracellular localization of *Cre-ER* protein following TM injection. Cre-ER protein was detected by using an anti-Cre antibody (D–F) in *CAGGCre-ER* embryos at 24 (E), or 48 h (F) postinjection (pi), of 3 mg TM. As an uninduced control, embryos were obtained from the mother injected with corn oil (D). Nuclei were visualized by DAPI (G–I). Initial blue color for DAPI staining was converted to green by Adobe Photoshop by maintaining the intensity intact to have better clarity for the level of colocalization. Merged pictures of two were shown in (A), (B), and (C), respectively. Note that without TM, Cre-ER protein is broadly distributed in cell (A, D, and G), but 24 h after injection of 3 mg TM, Cre-ER protein strongly localizes in nuclei (E). At 48 h postinjection, significant nuclear localization has almost disappeared (C, F, and I).

FIG. 5. The dynamic dose-related response to TM in *CAGGCre-ER^{TM}; R26R* mouse embryos. Compound transgenic embryos (*CAGGCre-ER^{TM}; R26R*) were subjected to whole-mount histochemical staining for β-galactosidase activity 6 (A, C) or 12 (B, D) h after administration of 3 (A, B) or 6 (C, D) mg of TM by intraperitoneal injection into the pregnant female. At 6 h postinjection (pi) of 3 mg/40 g body mass, TM-injected embryos showed very weak β-galactosidase activity (A), whereas embryos receiving 6 mg showed both a higher level of enzyme activity on the average and more cells with detectable activity (compare B with A and Fig. 2). At both concentrations, there was a significant increase in the recombination frequency 12 h postinjection, but recombination was significantly less than that observed 24 h postinjection (compare B and D with Fig. 2C).



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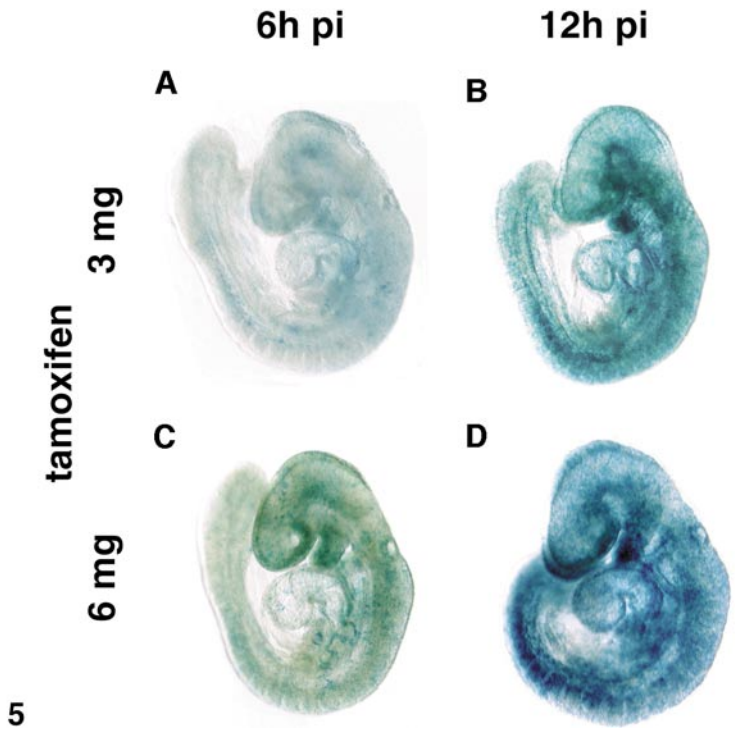

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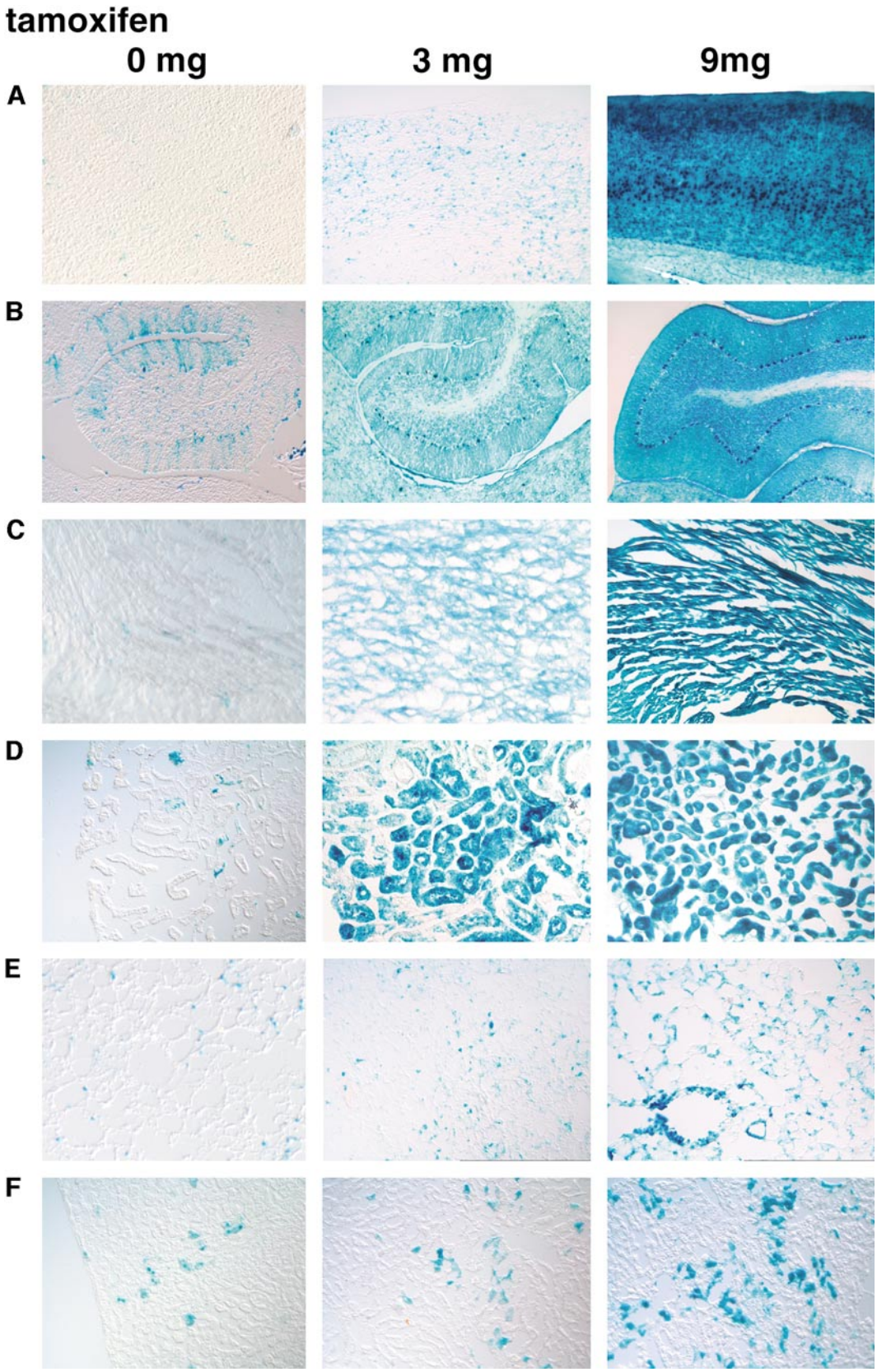


that TM induced a rapid, efficient recombination (Fig. 2F; and data not shown).

Dose-Dependent Recombination of CAGGCre-ER™ by TM in the Mouse Embryo

To evaluate the dose dependency of Cre recombinase activity in mouse embryos and the activity in different germ layer derivatives, the aforementioned protocol was followed with pregnant females receiving different doses of TM (0.5, 1, 1.5, 2, 3, 6, or 9 mg per 40 g of body weight). At all doses tested, TM-induced recombination was greater than the background recombination observed in *CAGGCre-ER™; R26R* embryos (compare Figs. 2B and 3A). Whole-mount visualization of embryos indicated a dose-dependent increase in the recombination efficiency in response to TM injections ranging from 0.5 to 3.0 mg with no apparent increase above 3 mg (Fig. 3A). Analysis of sectioned material revealed that recombination was observed in all germ-layer derivatives (heart, brain, surface ectoderm, and gut; Figs. 3B–3E), demonstrating the widespread expression of the *CAGGCre-ER™* transgene. The sectioned material also revealed a dose-dependent increase in the frequency of recombination 24 h postinjection up to the maximum dose of 9 mg of TM (Figs. 3B–3E). For example, in the telencephalon of embryos induced by a 3 mg injection of TM, approximately 75% of cells showed β-galactosidase activity, the percentage increasing to 85% of cells following a 9-mg injection. In addition, the proportion of cells within a tissue that showed weaker β-galactosidase activity, presumably reflecting a more recent recombination event than neighboring cells that display high levels of enzyme activity, decreased following an injection of 9 mg of TM. Thus, increasing the dose of TM from 3 to 9 mg results in a more rapid (high levels of enzyme activity in cells) and more efficient (ratio of recombined to nonrecombined cells [blue to white]) induction of recombination. Even so, a small percentage of cells in all tissues (estimated at less than 20%) failed to undergo recombination within 24 h of TM administration. However, when we examined embryos at 48 h postinjection, we observed a higher rate of recombination (95% in the telencephalon following a 3-mg injection at 8.5 dpc), indicating that the drug is present at effective concentrations beyond 24 h postinjection.

To attempt to address more directly the issue of how long *Cre-ER™* remains active, we examined the subcellular distribution of Cre-ER™ protein within embryonic tissues. We observed a significant nuclear accumulation of Cre-ER™ within 24 h of TM injection, which was dramatically reduced 48 h postinjection (Fig. 4). Thus, it is likely that the majority of Cre-ER™ activity is lost within 48 h of a single 3 mg/40 g injection of TM at 8.5 dpc, though it would require the introduction of a second reporter construct at these later stages to rigorously address this issue.

Next, we investigated how rapidly reporter gene expression could be detected within the embryo following injection of a single dose of TM (3 or 6 mg/40 g) at 8.5 dpc. Embryos were collected at 3, 6, and 12 h and β-galactosidase activity was assessed histochemically (Fig. 5; and data not shown). Enzyme activity was not detected 3 h postinjection, but low levels of activity were detected at 6 h, with slightly higher levels of activity in embryos receiving the higher dose of TM. Both the levels of β-galactosidase activity within cells and the number of cells with detectable enzyme activity markedly increased from 6 to 12 h postinjection (Fig. 5). Thus, recombination is initiated rapidly within the embryo, but it is likely that prolonged access to the drug is essential for high levels of recombination to be achieved.

Much of the analysis of this system has deliberately centered around 8.5 dpc as a time prior to the establishment of a functional placental connection when organogenesis and patterning of important tissues (for example, the neural tube) are initiating. To provide some additional insight into the effectiveness of TM-induced recombination at later stages of *in utero* development, single intraperitoneal injections of TM (3 and 9 mg) were administered at 11.5 and 14.5 dpc, and embryos were assessed by histochemical staining for β-galactosidase activity 24 h postinjection. As at earlier stages, widespread recombination was observed. At the lower dose, approximately 50–60% of cells underwent a detectable recombination event; this figure increased to 60–70% at the higher concentration (data not shown). Thus, this system provides a rapid, effective means by which gene activity can be modulated in the developing embryo at multiple stages of postimplantation development.

Induction of Cre Activity in Adult Mice

To test the potential use of the *CAGGCre-ER™* line in adult tissues, the recombination efficiency was examined following intraperitoneal injection of TM into 12-week-old animals. Organs were harvested from mice 5 days after 5 consecutive days of injection of 0, 3, or 9 mg of TM. Multiple injections of even the highest dose did not lead to

FIG. 6. Dose-dependent recombination in adult *CAGGCre-ER™; R26R* mice. Adult compound transgenic animals (*CAGGCre-ER™; R26R*) were injected once a day for 5 consecutive days with either corn oil alone or corn oil containing 3 or 9 mg of TM per 40 g body mass, as indicated. Frozen sections through various organs were assayed by histochemistry for β-galactosidase activity 5 days after the final injection. Nomarski views of (A) cerebral cortex, (B) cerebellum, (C) heart, (D) kidney, (E) lung, and (F) liver. Note that strong dose-dependent induction of recombination by TM was observed in the brain, heart, kidney, and lung. Only low levels of recombination are evident in the liver.

any obvious change in the behavior, feeding, or appearance of the mice over the period of study. As expected from the slight "leakiness" of Cre-ER^{TM} activity that was observed at embryonic stages, β-galactosidase activity was detected in some cells in the absence of TM injection: however, the proportion of these cells remained low, indicating the tight regulation of Cre activity over many weeks and many rounds of cell division. Efficient dose-dependent recombination was observed in most organs, including the cortex (Fig. 6A), cerebellum (Fig. 6B), and hippocampus in the brain (data not shown), heart (Fig. 6C), kidney (Fig. 6D), lung (Fig. 6E), pancreas, and intestine (data not shown). In all cases, recombination was observed in a broad range of cell types. In contrast, the liver displayed a highly mosaic pattern of recombination that was dose-dependent as in the other organs (Fig. 6F). This mosaic pattern of recombination is not due to a problem in reporter expression within the liver, as the locus is expressed ubiquitously in this tissue (data not shown). These results indicate that the transgenic line allows efficient manipulation of gene activity in many, but not all, organs. To address how rapidly recombination is detected in adult tissues, we assayed β-galactosidase activity in a variety of tissues 24 h after a single, intraperitoneal injection of 9 mg of TM injection into an adult (40 g) mouse (Fig. 7). Although recombination was detected in many tissues, recombination was highly mosaic, being most readily detected in highly vascular tissue (e.g., the heart). Thus, either a single dose, or a 24-h period postinjection is not sufficient to lead to as widespread a modification of gene activity, as was observed following the multiple injection scheme discussed earlier. To determine whether this simply reflected the period of exposure to the drug, we also examined tissues 5 days postinjection (Fig. 7). An increase in recombination was observed in some tissues (cortex and kidney; Fig. 7), but generally lower levels of recombination were observed in most tissues when compared with multiple administrations of TM (Fig. 6).

Dose-Dependent Recombination in Cultured PEF Cells

As an alternative to the manipulation of gene activity in the whole organism, we investigated the ability of 4OH-TM to modulate recombination in cell cultures derived from 14.5-dpc embryos. Fibroblast cultures were prepared and recombination assayed 24–48 h following the addition of varying concentrations (10 pM to 1 μM) of 4OH-TM. All doses resulted in a small increase in activity over 24 h (4- to 5-fold) and a large increase 48 h (13- to 27-fold) after the initiation of drug treatment (Fig. 8A). The large increase in activity correlated with a substantial increase in the number of cells displaying β-galactosidase activity (Fig. 8B). Indeed, 75% of all cells underwent a recombination event within 48 h in response to the lowest dose of TM tested (10 pM; Fig. 8B), and this number increased to over 90% at the highest concentration (1 μM; Fig. 8B). Thus, the *CAGGCre-ER^{TM}* line is an effective tool for manipulating gene activity in either the organism or in cell culture.

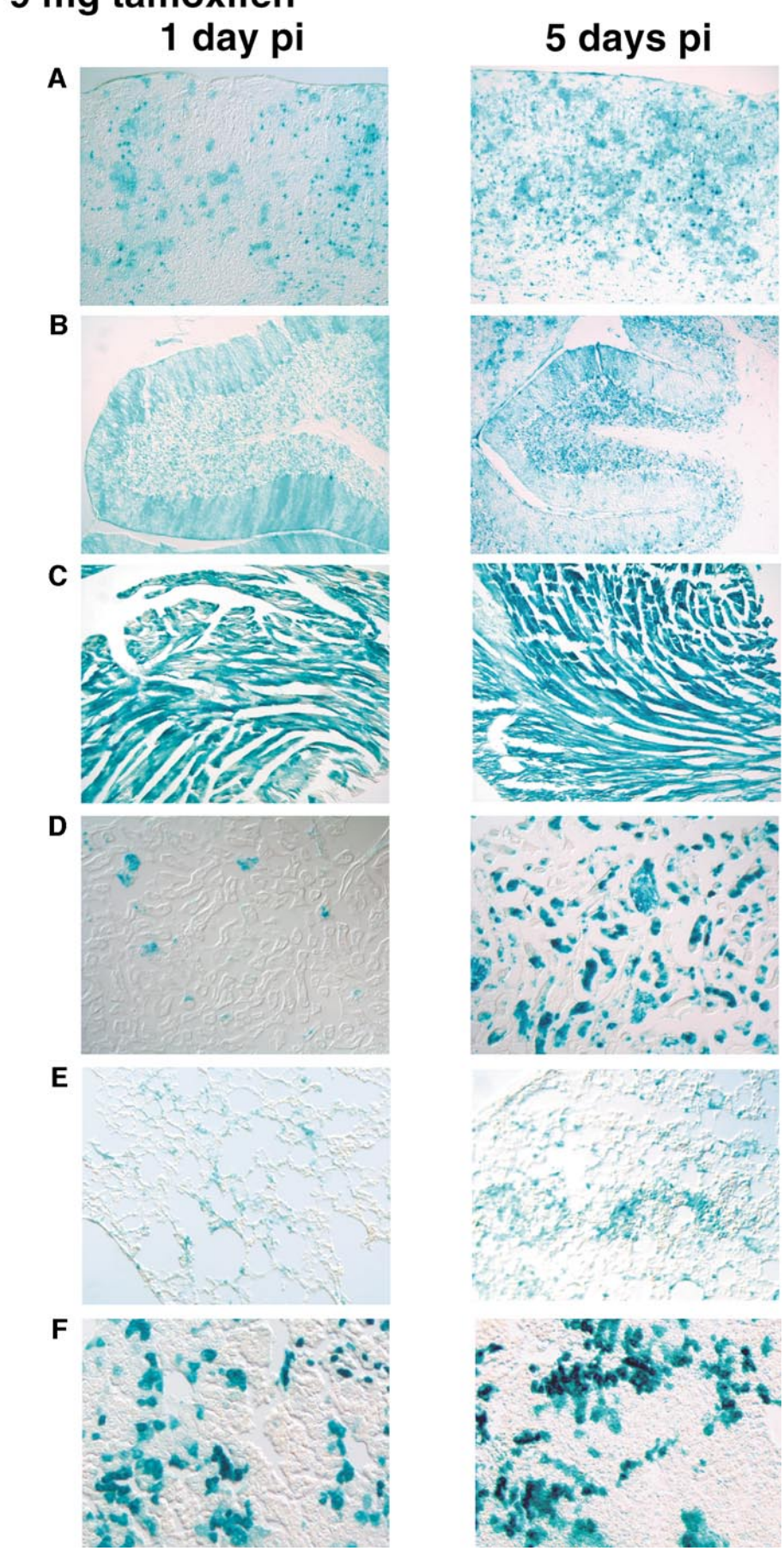
DISCUSSION

Dose-Dependent Recombination of CAGGCre-ER^{TM}

We have generated a transgenic mouse line that exhibits widespread expression of a TM-inducible Cre transgene: addition of TM or its derivative 4OH-TM results in efficient, dose-dependent recombination in embryos developing *in utero*, in adult mice, and in cell culture. A recent report (Vooijs *et al.*, 2001) describes a similar approach in which a Cre-ER^{TM} transgene was "knocked-into" the ROSA26 locus. These authors demonstrated effective recombination in a variety of adult tissues as monitored by Southern analysis of whole tissue extracts 7 days after a multiple round of TM injection as we report with the *CAGGCre-ER^{TM}* transgene. However, Vooijs *et al.* (2001) did not address the effectiveness of their line for *in utero* modification of gene activity. Thus, a strict comparison of the relative effectiveness of each strain in a similar analysis is not possible at this time, but the general availability of two independent strains that affect the temporal modification of gene activity will be a valuable asset to the research community.

Given our own interests, we have focused for the most part on embryos between 8.5 and 9.5 dpc, when initial patterning events have established the general organization of the body plan, but coincident with, or just prior to, the establishment of most of the organ primordia. In an earlier study in which expression of the same transgene was restricted to the neural tube under the control of a *Wnt1* regulatory element, we failed to observe efficient recombination during this particular period. However, efficient recombination was observed after the establishment of an embryonic/maternal circulation 1 day later (Danielian *et al.*, 1998). The current work indicates that establishment of a circulatory connection is not essential and that the absence of recombination in the *Wnt1* model most likely reflects a lower level of expression of the Cre transgene under *Wnt1* control or a changes in the dosing scheme adopted here.

The *CAGGCre-ER^{TM}* line displayed dose-dependent recombination between 0.5 and 9 mg of TM, with higher doses giving higher degrees of recombination. However, the recombination efficiency has to be weighed against the lethality (presumably due to a disruption of maternal–fetal interactions at high concentrations of the estrogen-like steroid) that results from administration of high concentrations of TM (Sadek and Bell, 1996). In this study, we focused on animals of a particular body weight (40 g) because of preliminary evidence that the toxic effects of TM concentration on embryonic development did not follow a simple linear progression with increasing body mass of the pregnant mouse. Little toxicity was observed following injection of 2 mg or less: however, significant lethality was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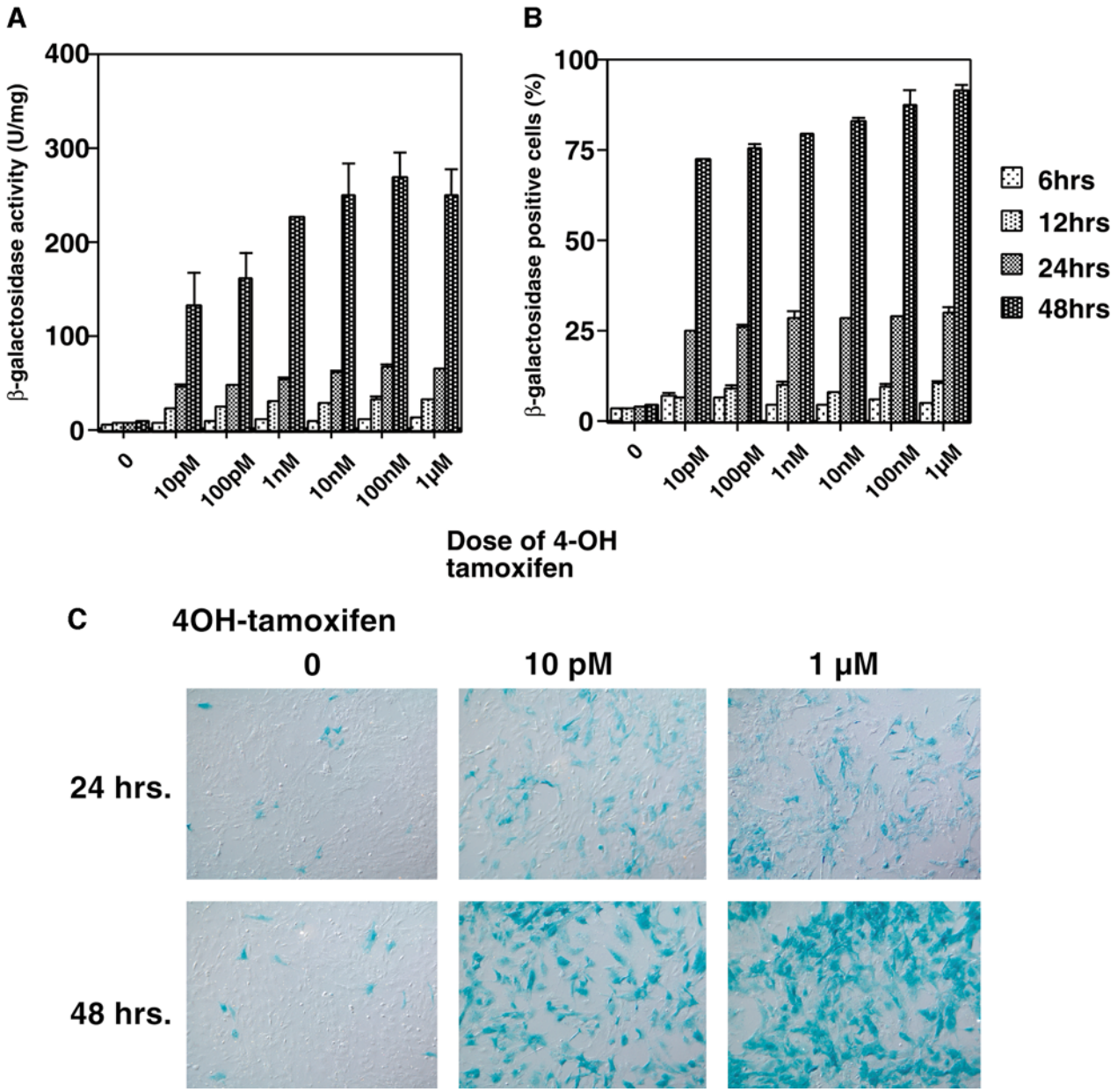

FIG. 8. 4OH-TM induces efficient recombination in cultured primary embryo fibroblasts (PEF). PEF cells were isolated from 14.5-dpc compound transgenic embryos (*CAGGCre-ER^{TM}; R26R*) and treated with different doses of 4OH-TM as indicated, and the β-galactosidase activity was determined by using a biochemical assay of cell lysates (A) or by direct counting of cells after histochemical detection (B). Each point is the mean of triplicate measurements with the standard deviations indicated by the error bars. (C) Nomarski views of the histochemical detection of β-galactosidase activity in cells following treatment with 0 or 10 pM or 1 μM 4OH-TM for 24 or 48 h.

FIG. 7. Recombination efficiency following a single injection of TM into adult mice. Adult compound transgenic animals (*CAGGCre-ER^{TM}; R26R*) were injected with 9 mg of TM per 40 g of body mass. One day or 5 days postinjection (pi), a variety of organs were harvested, frozen sections were prepared, and this material was subjected to histochemistry to assess β-galactosidase activity. Nomarski views of (A) cerebral cortex, (B) cerebellum, (C) heart, (D) kidney, (E) lung, and (F) liver.

observed following injection of 3–9 mg. At 3 mg, a dose that leads to over 50% of cells undergoing a recombination event 24 h post injection, most embryos will survive to term, though a cesarean section is required for delivery. In contrast, most embryos developing in females receiving 6 or 9 mg of TM survive to 13.5 dpc, but none survived to term. At this dose, up to 80% of cells within a tissue underwent recombination within 24 h of TM injection. Consequently, if rapid, efficient recombination was required to study the properties of a particular gene in a narrow time interval following the recombination-mediated genetic modification, injection of the highest doses would seem to be a reasonable strategy. If, in contrast, later survival is important, injection of 3 mg of TM per 40 g of body weight would appear to be the best option. It should be noted that our measure of recombination is the presence of β-galactosidase activity. This requires transcription and translation of the reporter allele following the recombination event. Consequently, the actual recombination frequency at the DNA level at any time is likely to be higher. Further, if one examines the intensity of histochemical staining of cells displaying β-galactosidase activity, it is evident that the ratio of cells exhibiting weak versus strong activity decreases as the TM injection dosage is increased from 3 to 9 mg. The most likely explanation is that weak activity reflects a more recent recombination event and consequently β-galactosidase levels are not maximal in the cell. Indeed, consistent with this view, embryos harvested at 48 h postinjection of 3 mg of TM displayed a higher percentage of cells (95%) exhibiting detectable β-galactosidase levels. The reduction in nuclear localized Cre-ER protein 48 h after the injection of 3 mg of TM suggests that further recombination is less likely beyond this time point. Finally, the recombination-inducing activity and developmental toxicity of a specific dosage are likely to vary at different stages of pregnancy. A thorough investigation of responses at different stages awaits further study, but our results indicate that injection of 3 mg at 10.5 dpc leads to complete recombination in most tissues by 12.5 dpc and that administration of the highest dose of TM at 11.5 and 14.5 dpc induces a robust recombination in many tissues within 24 h (greater than 50%). Further, injection of the highest dose (9 mg) is compatible with survival to term.

As a further demonstration of the utility of this line, we showed that multiple rounds of intraperitoneal injection of TM (3 mg/day for 5 days) resulted in efficient induction of recombination in a broad spectrum of adult tissues, including the brain, indicating that the blood–brain barrier does not prevent access of the drug. Clearly not all tissues respond equally well; for example, although the liver expresses the ROSA26 reporter quite strongly in control experiments, we observe only patchy activity following TM injection, indicating very poor levels of recombination. It is unlikely that this reflects a problem in the accessibility of the drug to this tissue as the liver actually converts TM to 4OH-TM, the actual steroid that binds *in vivo* to the mutated ER domain (Littlewood *et al.*, 1995; Williams *et al.*, 1994). A more likely possibility is varying levels of *CAGGCre-ER*^{TM} expression in adult tissues, though this remains to be determined.

Potential Use of CAGGCre-ER^{TM}

With the adjustment of TM dose to balance recombination efficiency versus toxicity, this line could be particularly useful in controlling gene activity in a temporally regulated fashion. For example, if some essential sequence within a gene is flanked by two loxP sites (gene A in Fig. 1A), TM administration can be used to remove activity of this gene at a certain stage of embryonic or postpartum development. Alternatively, if the sequence following the second loxP site encodes a functional gene (gene B in Fig. 1A), this protocol would allow conditional activation of the gene. This approach should allow researchers to circumvent problems associated with germ-line ablation and early embryonic lethality caused by a standard knockout or overexpression approaches, thereby allowing a more complete analysis of gene function.

Clearly, widespread expression of the transgene will ensure a general removal or activation of a given gene in a broad spectrum of tissues at a given stage of development. In many circumstances, having a precise temporal control of the recombination event will circumvent the problem of genes that may have unique activities in different tissues at different stages of development. However, the situation is more complex when the given gene may play several different roles at the same stage of development. Here, tissue-specific removal of gene activity using tissue-specific transgenic Cre lines provides a complementary tool. Clearly, the combination of tissue-specific regulatory element and *Cre-ER*^{TM} provides a valuable alternative for the dissection of sequential actions of a gene within a given tissue.

By controlling the site of drug delivery, the CAGGCre-ER^{TM} transgene may also allow spatial modulation of gene activity. Local injection of 4OH-TM has been shown to activate other forms of TM-inducible Cre (Feil *et al.*, 1997; Vooijs *et al.*, 2001; Indra *et al.*, 1999; Vasioukhin *et al.*, 1999), providing a spatial restricted response. Further, if combined with the previously developed techniques of ultrasound backscatter microscopy (UBM), which allows visualization of embryos *in utero* (Gaiano *et al.*, 1999), or stereotaxic injection, the potential application of this line could be greatly broadened.

The demonstration that cells can be removed from *CAGGCre-ER*^{TM}*; R26R* embryos and their genetic activity manipulated in cell culture suggests a number of approaches. For example, new cell lines can be created directly from embryonic or adult tissues of a particular genetic make-up in which the presence of *CAGGCre-ER*^{TM} will enable the *in vitro* manipulation of gene activity in the precise study of a particular cellular action. In *vitro* explants also provide another means by which the response may be localized to a

given tissue or organ at a specific stage of development. Finally, by culturing preimplantation embryos in the presence of 4OH-TM, it may be possible to generate large numbers of embryos in which gene activity is modified at an early stage.

The fact that CAGGCre-ER™ induces dose-dependent recombination suggests a potential use of this line in the generation of genetic mosaic animals, an extremely valuable research tool in the study of invertebrate model systems. Low doses of TM induce recombination in a small fraction of cells. By regulating the dose of TM, one could generate different levels of mosaicism. With a means to identify cells that have undergone the recombination event, the properties of these cells can be investigated and compared with wild-type neighbors. Further, it may be possible to generate a phenotypic series if the levels of a certain factor can be controlled by altering the numbers of cells that express that factor. Generation of genetic mosaics is also a generally useful technique to study the function of genes, when ablation or overexpression of these genes causes lethality. Use of the *CAGGCre-ER™* mouse will provide a complementary approach to more classical chimeric experiments that use genetically distinct embryo/embryo or embryo/ES cell chimeras (Nagy and Rossant, 2001).

Another potential application of the pCAGGCre-ER™ line is for clonal analysis. The key to this approach is that recombination should occur very infrequently so that two cells that share reporter gene expression lying close to one another are more likely to represent clonal relatives than independent recombination events. Others have used rare mitotic recombination events to label cells (Bonnerot and Nicolas, 1993; Mathis *et al.*, 1997; Sanes, 1994). The TM-independent leaky recombination we observe may provide an alternative approach. This recombination event clearly occurs relatively late in development, as evidenced by the small number of cells displaying β-galactosidase activity at 9.5 dpc (less than 0.1%) and later stages, and their sporadic distribution which varies from embryo to embryo. Interestingly, despite the low frequency of marked cells, many are clustered, suggesting that they are clonally related (see Fig. 2B). Further, during the screening of *CAGGCre-ER™* founders, we identified a second transgenic line (line 37) which showed a highly patchy distribution of cells with β-galactosidase activity scattered throughout the embryo following TM injection (data not shown). Modulating the activity of this line with different TM concentrations may allow clonal analysis to be optimized.

Although genetic studies in the mouse have played a major role in dissecting developmental pathways, their potential for addressing issues of physiology in the adult mouse have yet to be fully realized. Clearly, one important reason for this is that many genes that regulate the adult physiological state also have essential functions during embryonic development precluding the generation of adult mutants without conditional gene-removal strategies. One particularly valuable role that this transgene will play is in genetic modification in the adult mouse. Although it may not be possible to induce complete recombination, recombination frequencies for most tissues are very high with the dosing schedule we describe. This should enable the identification of new modulators of adult physiology and the generation of valuable disease models for the study of adult pathologies, including cancer. Finally, although not elaborated here, the *CAGGCre-ER™* line should be useful in all systems where the enzymatic activity of Cre at target *lox P* sites has previously been applied to manipulating inversion, integration, and translocation of chromosomes (Nagy, 2000). In summary, the approach we describe will be a valuable new tool in dissecting the mechanisms underlying developmental, physiological, and pathological processes.

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